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Original Article

A multiplex assay of *Trichomonas vaginalis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections in genital specimens

Mahmoud Nateghi Rostami[1,2], Batool Hossein Rashidi[3], Fatemeh Aghsaghloo[4], Azam Habibi[5]

[1] *Department of Microbiology and Immunology, Faculty of Medicine, Qom University of Medical Sciences, Qom, Iran*
[2] *Department of Parasitology, Pasteur Institute of Iran, Tehran, Iran*
[3] *Department of Obstetrics and Gynecology, Vali-Asr Reproductive Health Research Center, Tehran University of Medical Sciences, Tehran, Iran*
[4] *Department of Microbiology, Islamic Azad University, Qom Branch, Qom, Iran*
[5] *Department of Microbiology, Science and Research Branch, Islamic Azad University, Arak, Iran*

Abstract

Introduction: A significant proportion of patients with Sexually Transmitted Infections (STIs) are coinfected with multiple pathogens. We report here development of a multiplex PCR for simultaneous detection of *Chlamydia trachomatis* (*C.trachomatis*), *Neisseria gonorrhoeae* (*N.gonorrhoeae*) and *Trichomonas vaginalis* (*T.vaginalis*) in genital specimens from women.

Methodology: After detection of the organisms by routine techniques including PCR, culture and direct smear, multiplex-PCR was optimized to detect *ompI* gene of CT, *parC* of NG, ITS ribosomal RNA of TV as target genes. The limit of detection (LOD) was determined using serially diluted genomic DNA from known number of each pathogen.

Results: Totally 300 volunteers with mean age of 36.5 ±7.03 years were included and 266 (88.7%) had genitourinary clinical manifestations. Of 300 women, 150 (50.0%) were infected. Of them, 17 (5.7%) had *N. gonorrhoeae*, 98 (32.7%) *T. vaginalis* and 35 (11.7%) *C. trachomatis*. Multiplex-PCR revealed a total of 10 coinfections (3.3%) including 2 specimens of *C. trachomatis/ N. gonorrhoeae*, 3 specimens of *C .trachomatis/ T. vaginalis* and 5 specimens of *N. gonorrhoeae*/*T. vaginalis* coinfections. The sensitivity and specificity of multiplex-PCR for detecting *N. gonorrhoeae* were 100% and 98.59% (279 of 283) respectively and, for *C. trachomatis* and *T. vaginalis* were 100%. The LOD was 0.1 pg of DNA for *C. trachomatis* and *N. gonorrhoeae*, and 1.5 pg for *T. vaginalis.*

Conclusions: The performance of this multiplex-PCR makes it a sensitive, rapid and affordable technique in clinical laboratory for simultaneous detection of STIs.

Key words: Multiplex PCR; *Chlamydia trachomatis*; *Neisseria gonorrhoeae*; *Trichomonas vaginalis*.

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Introduction

According to the estimate of the World Health Organization [1], each year about 500 million people become affected by one of 4 sexually transmitted infections: chlamydiosis, gonorrhea, syphilis and trichomoniasis. More than 1 million people acquire a STI every day, with the largest proportion in the region of south and southeast Asia [2]. More than 30 bacterial, viral and parasitic STI pathogens, *T. vaginalis* and *C. trachomatis* are the most common and curable sexually transmitted pathogens worldwide [3]. STIs can have serious complications beyond the usual presentations of the infection such as trasplacental transmission of infection, facilitation of HIV transmission, ectopic pregnancy, stillbirth, and pelvic inflammatory disease (PID), one of the major causes of infertility [4,5]. However, most of the women with genital infections are asymptomatic and early detection of STIs would be useful to prevent serious complications. WHO supports development of rapid and low-cost point-of-care diagnostic tests as new approach for prevention of STIs [1]. The purpose of reporting STIs is to ensure that infected patients are quickly diagnosed and appropriately treated. Evidences show that in areas where STIs screening programs were implemented for sexually active women, the rate of infections and their complications were decreased [3]. Over the past years, the diagnosis of STIs has been largely dependent on traditional techniques such as culture, stained smear and enzyme immunoassay [6]. These methods are usually time consuming and labor-intensive and lack convincing sensitivity for definite identification of STIs

in symptomatic and asymptomatic patients. With the advent of technologies such as nucleic acid amplification (NAAT), major improvements in the ability to detect these STIs have occurred [7-10]. PCR-based tests have generally been more sensitive than traditional tests in diagnosis of *C. trachomatis* and *N. gonorrhoeae* [11-14]. Several studies have shown that a significant proportion of patients are coinfected with two STIs simultaneously [15,16]. With the annual incidence of about 500 million cases of STIs, significant rate of coinfections is conceivable which justify development of methods to diagnose multiple genital infections in simple steps. In addition, identification of more than one agent in one specimen is more cost-effective, more rapid, and more acceptable for the patients. Although multiplex PCR has been applied to the detection of multiple viruses and bacteria in clinical specimens [17,18] this approach has less been established for simultaneous detection of prokaryotic and eukaryotic STIs [19]. We report here the development of a multiplex PCR for the simultaneous detection of *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* in genital swab specimens collected from women.

Methodology

Study population, ethical considerations and sampling

The study was approved by Ethical Committee of Tehran University of Medical Sciences (TUMS) Tehran, Iran. All procedures were in accordance with the ethical standards of human experimentation of 1964 Declaration of Helsinki and its later amendments. From May 2014 to April 2015 women who admitted to Gynecology Hospital and Health Clinics of Qom city, central Iran, because of genital complaints were recruited for the study after provision of written consent and willingness to participate. Inclusion criteria included age range between 18 to 60 years and no administration of systemic or topical antibiotics within last 1 month. After examination of each volunteer by a gynecologist, demographic data, history and clinical signs/symptoms were recorded in a questionnaire.

Four swab samples were collected from each volunteer under aseptic conditions. First endocervical swab was cultured on Thayer Martin (TM) agar (Merck, Darmstadt, Germany) for detection of *Neisseria*, second vaginal swab was used for smear preparation for detection of *Trichomonas*, and two swabs from vaginal and endocervical canals were transferred to sterile PBS, pH 7.2, mixed and used for DNA extraction.

DNA extraction

DNA extraction was carried out on the vaginal/endocervical samples by using AccuPrep Genomic DNA Extraction Kit (BIONEER, Daejeon, South Korea). Briefly, 1.5 ml sample tubes were centrifuged 5 minutes at 7000 rpm, then 20 µl of proteinase K was added to the pellet followed by adding 200 µl of GC binding buffer and tubes were incubated at 60˚C water bath for 10 minutes. Then 100 µl of absolute isopropanol was added and the contents were transferred to binding tubes. Tubes were washed with 500 µl of W1 washing buffer at 8000 rpm for 1 minute then with W2 washing buffer at 12000 rpm for 1 minute. Finally, 50 µl of elution buffer was added to the tubes and centrifuged at 8000 rpm for 1 minute and elutes were collected as DNA and kept at -20˚C until use. In some instances DNA extraction was done by phenol-chloroform method as described [20].

Detection of C. trachomatis infection

For molecular identification of *C. trachomatis* in genital specimens*, ompI* target gene encoding for chlamydial major outer membrane protein (MOMP) was amplified by nested PCR as previously described [21]. The sequence of external primers (Ex-p1/Ex-p2) and nested primers (In-p1/In-p2) are shown in Table 1 (CinnaGene, Tehran, Iran). *C. trachomatis* ATCC VR-878D DNA was used as standard.

Detection of N. gonorrhoeae infection

N. gonorrhoeae strain was cultured and identified by biochemical reactions [22]. For molecular detection of *N. gonorrhoeae*, NG.F/NG.R primer pair, which amplify *ParC* gene encoding for topoisomerase IV enzyme of the organism, was used (CinnaGene, Tehran, Iran) (Table 1). Amplification was carried out in a total volume of 25 µl containing 2.5 µl 10× PCR buffer, 1.5 mM $MgCl_2$, 0.5 µl of each primer (25 pmol/ml), 0.2 mM of each dNTPs, and 1 U *Taq* DNA polymerase (Fermentas Life Sciences, York, UK). The conditions for *N. gonorrhoeae* PCR were as follows: initial denaturation of 94°C for 4 minutes, then 30 cycles of 94ºC for 60 seconds, 53.8ºC for 45 seconds, 72°C for 45 seconds, followed by final extension of 72ºC for 10 minutes. Positive results were further confirmed by real-time PCR assay targeting the *porA* pseudogene as previously described [23]. Sequences of primer pair NIS.F and NIS.R were shown in Table 1 (CinnaGene, Tehran, Iran). *N. gonorrhoeae* ATCC 49226 was used as a standard reference strain.

Detection of T. vaginalis infection

Wet mount smears were examined for identification of motile flagellated trophozoite of *T. vaginalis* immediately after sampling. For molecular detection of *T. vaginalis*, TV.F/TV.R primer pair (CinnaGene, Tehran, Iran), which amplify *ITS* ribosomal RNA gene was used (Table 1) following the protocol as previously described [23]. For confirmation of the results, some *T. vaginalis* PCR products were submitted for sequencing.

Multiplex PCR assay

Primers with acceptable physicochemical parameters as analyzed by GeneRunner program Ver. 3.05 (Hastings software Inc., NY, USA) were selected and aligned with the sequence from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) to test for possible nonspecific interactions with other organisms. BioEdit Sequence Alignment Editor software ver. 7.0.4.1 (Tom Hall, Carlsbad, USA) was used to align gene sequences from different strains to identify conserved regions for designing specific primers. The specific primers used to amplify the 3 target organisms were Ch.F/Ch.R, NG.F/NG.R and TV.F/TV.R pairs as shown in Table 1.

The specificity of the assay of each primer pair was tested with common eukaryotes and Gram-positive and Gram-negative bacteria which representing normal vaginal flora. Suspensions of bacteria adjusted to 0.5 McFarland standard (equal to 1.5×10^8 colony-forming units (CFU)/mL) were prepared. Genomic DNAs were extracted from: *Candida albicans, Escherichia coli, Gardnerella vaginalis, Lactobacillus acidophilus, Pseudomonas aeruginosa, Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus agalactiae,* and *Enterobacter aerogenes.*

To determine the limit of detection (LOD) for each pathogen, genomic DNA was extracted from known number of *C. trachomatis, N. gonorrhoeae* and *T. vaginalis,* then was 10-fold serially diluted and spiked into the reactions then multiplex PCR was performed.

Multiplex PCR conditions were established by using a mixture of DNA from all three organisms. The 25 µl PCR reaction mixture consisted of 67 mM Tris-HCl; pH 8.8, 0.01% Tween 20, 3 mM $MgCl_2$, 2.5 mM each dNTPs, 3 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA) and 10 µM of each specific primer pair. The target DNA concentrations were adjusted to 1-3 µl (100 ng) depending on the organism. Annealing temperatures ranging from 50°C to 62°C were tested and after several adjustments it was optimized at 56°C. Once the conditions were optimized, genital swab samples from 300 women were subjected to multiplex PCR assay. The amplified products were electrophoresed on 1.5% agarose gels containing ethidium bromide in buffer.

For determining the performance of multiplex PCR, a combination of molecular and conventional methods was considered as gold standard for each pathogen.

Results

In this study, a total of 300 women volunteers were included. Mean ±SD age of the volunteers was 36.5±7.03 years. Of total 300 women, 266 (88.7%) had clinical signs/symptoms such as lower abdominal pain (182; 60.7%), malodorous discharge (123; 41.0%), genital itching (115; 38.3%), and painful intercourse (111; 37.0%). Pregnancy-related complications included history of curettage (45; 15.0%), abortion (66; 22.0%), ectopic pregnancy (3; 1.0%), premature neonate (10; 3.3%), still birth (8; 2.7%), infertility (23; 7.7%) and premature rupture of membranes (PROM) (32; 10.7%).

In evaluation of primer pairs, no significant similarities between primer sequences and any of the tested genomes, which could theoretically lead to non-

Table 1. Characteristics of primer pairs used for identification of infections in genital specimens.

Organism	Target gene	Primers	Product size (bp)
C. trachomatis	MOMP PCR	Ch.F 5'-CCTGTGGGGAATCCTGCTGAA-3' Ch.R 5'-GTCGAAAACAAAGTCACCATAGTA-3'	240
	ompI	Ex-p1: 5'-ATGAAAAAACTCTTGAAATCGG-3' Ex-p2: 5'-TTTCTAGATTTTCATCTTGTT-3'	1100
	nested-PCR	In-p1 5'-TTTCTAGATTTTCATCTTGTT-3' In-p2 5' CCTTGCAAGCTCTGCCTGTGGGGAATCCT-3'	1000
N. gonorrhoeae	*ParC*	NG.F: 5'-GTT TCAGACGGCCAAAAGCC-3' NG.R: 5'-GGCATA AAATCCACCGTCCCC-3'	331
	ProA real time-PCR	NIS.F 5'-CGGTTTCCGTGCGTTACGA-3' NIS.R 5'-CTGGTTTCATCTGATTACTTTCCA-3'	131
T. vaginalis	ITS ribosomal RNA	TV.F: 5'- TAGGTGAACCTGCCGTTGG-3' TV.R: 5'-AGT TCAGCGGGT CTTCCTGCG-3'	361

Table 2 Frequency of STIs in genital swab specimens from studied women.

Infection		Frequency	subtotal
Chlamydia trachomatis	Single infection	30 (10.0%)	35 (11.67%)
	Multiple infection	*with NG* 2 (0.67%) *with TV* 3 (1.0%)	
Neisseria gonorrhoeae	Single infection	10 (3.34%)	17 (5.67%)
	Multiple infection	*with CT* 2 (0.67%) *with TV* 5 (1.67%)	
Trichomonas vaginalis	Single infection	90 (30.0%)	98 (32.67%)
	Multiple infection	*with CT* 3 (1.0%) *with NG* 5 (1.67%)	
Negative specimens		150 (50.0%)	
Total		**300 (100.0%)**	

specific amplification, were found. Results showed no cross reactivity between target sequences and common vaginal flora in multiplex PCR. The LOD was 0.1 pg of DNA (equivalent to approximately 20 copies) for *C. trachomatis* and *N. gonorrhoeae*, and 1.5 pg (equivalent to approximately 1-2 copies) for *T. vaginalis.*

In detection of genital infections, single or multiple infections were shown in 150 (50.0%) volunteers, and the rest had negative results. Totally *N. gonorrhoeae* infection was detected in 5. 7.0% (17/300) of the specimens. *N. gonorrhoeae* was identified in 21 specimens by PCR. However, in real-time PCR assay 17 specimens provided positive results for *N. gonorrhoeae* and 4 false PCR-positive specimens were negative in real-time PCR and identified as other *Neisseria* spp. In multiplex PCR 21 (7.0%) specimens showed positive results for *N. gonorrhoeae* including 2 (0.7%) mixed infection with *C. trachomatis* and 5 (1.7%) mixed infections with *T. vaginalis* (Table2). Four false positive specimens had no mixed infections.

Totally *T. vaginalis* infection was detected in 32.7% (98/300) of the specimens. *T. vaginalis* was positive in 34 (11.3%) specimens in wet mount examination. *T. vaginalis* infection was identified in 96 (32.0%) specimens by using conventional PCR. In multiplex PCR 98 (32.7%) specimens showed positive results for *T. vaginalis* including 3 mixed infections with *C. trachomatis* (1.0%) and 5 mixed infections with *N. gonorrhoeae* (1.7%) (Table2). Sequence data of PCR products of *T. vaginalis* ITS ribosomal RNA gene were submitted to GenBank under the accession number KP221674.1.

Totally 11.7% (35/300) of specimens showed positive results for *C. trachomatis* infection. *C. trachomatis* was identified in 35 (11.7%) specimens in *ompI* nested-PCR assay. By using multiplex-PCR, *C. trachomatis* was identified in 35 (11.7%) specimens including 2 mixed infection with *N. gonorrhoeae* (0.7%) and 3 mixed infections with *T. vaginalis* (1.0%) (Table2).

In the next step, the multiplex PCR assay was established with the annealing temperature of 56°C, in which all three amplicons were efficiently generated. Multiplex PCR revealed a total of 10 mixed double coinfections comprising 3.34% of the total specimens (Table 2). None of genital specimens showed infection with all three organisms simultaneously.

All *T. vaginalis* positive specimens with conventional PCR as well as two other specimens were positive in multiplex PCR (98/98). Multiplex-PCR method showed 100% sensitivity and 100% specificity in detection of *T. vaginalis* infection in vaginal specimens (Table 3). Multiplex PCR detected all 17 *N. gonorrhoeae* true positive specimens. Four specimens of other *Neisseria* spp. were falsely positive with multiplex assay. Multiplex PCR method showed 100%

Table 3 Sensitivity and specificity of different methods used for detection of STIs.

Organism	Method	Sensitivity % (no. of true positive/ no. of infected persons)	Specificity % (no. of true negative/ no. of non-infected persons)
Chlamydia trachomatis	nested-PCR	100% (35/35)	100% (265/265)
	mPCR	100% (35/35)	100% (265/265)
Neisseria gonorrhoeae	Conventional PCR	100% (17/17)	98.59% (279/283)
	Real time PCR	100% (17/17)	100% (283/283)
	mPCR	100% (17/17)	98.59% (279/283)
Trichomonas vaginalis	Wet mount	34.69% (34/98)	100% (202/202)
	Conventional PCR	97.96% (96/98)	100% (202/202)
	mPCR	100% (98/98)	100% (202/202)

sensitivity and 98.59% specificity in detection of *N. gonorrhoeae* infection in endocervical specimens. Multiplex PCR detected 35 of 35 endocervical specimens with *C. trachomatis* infection. Multiplex PCR method showed 100% sensitivity and 100% specificity in detection of *C. trachomatis* infection in endocervical specimens.

Discussion

Several reports have shown that coinfections of STIs are common in women [15, 16]. We have recently reported the significant prevalence of STIs in genital samples of the women of Qom, central Iran [23]. Application of conventional PCR is a common strategy for detection of STIs, which is adopted by many laboratories in Iran. However, collection of genital samples for diagnosis of each STI often causes discomfort for the female patients, the principal advantage of the multiplex PCR described here is simultaneous detection of 3 common infections in one reaction. In the current study, 10 specimens had DNA from two pathogens, with the highest rate for mixed infections of *N. gonorrhoeae* with *T. vaginalis*. The sensitivities of multiplex PCR for detecting *C. trachomatis*, *N. gonorrhoeae* and *T. vaginalis* were 100%. The specificities were between 98.6%-100% for all the three organisms.

The performance of multiplex PCR is affected by several factors such as the type of specimen and clinical characteristics of the patients. Although use of urine samples provides a noninvasive approach to the diagnosis of STIs in men, but in women genital swab is usually the preferred specimen for diagnosing STIs. Mahony and colleagues developed a multiplex PCR assay for the simultaneous detection of *C. trachomatis* and *N. gonorrhoeae* in first void urine specimens. They reported the sensitivity of multiplex PCR for detecting *C. trachomatis* was 100%, compared with 81.8% for enzyme immunoassay and for detecting *N. gonorrhoeae* was 92.3% compared with 84.6% for urethral culture and the specificity was 100% for both *C. trachomatis* and *N. gonorrhoeae* [24]. In comparison, our multiplex PCR has a similar sensitivity/specificity for detection of *C. trachomatis* but higher sensitivity for detection of *N. gonorrhoeae* on endocervical specimens. Several sensitive multiplex assays for the simultaneous detection of common STIs from various specimen sources have been introduced which utilize automated systems or real-time PCR platform [19,25,26]. However, our multiplex assay is not laborious and such an assay is an affordable method that can be performed on different laboratory settings in developing countries. In this study, coinfection rates of two pathogens were from 0.7% to 1.7%. Similar to our findings, another study from United States of America performed with a cohort of more than 7500 urogenital samples revealed prevalence of *T. vaginalis*, *C. trachomatis*, and *N. gonorrhoeae* as 8.7%, 6.7%, and 1.7% respectively, coinfection rates 1.3% for *T. vaginalis*/*C. trachomatis*, 0.61% for *N. gonorrhoeae*/*C. trachomatis,* and 1.3% for *T. vaginalis*/*N. gonorrhoeae* [15]. We found two coinfections of *C. trachomatis* with *N. gonorrhoeae* among 300 endocervical specimens. Higher rates of coinfection of *C. trachomatis* with *N. gonorrhoeae* in urine samples of women or men with urethritis were reported [27,28]. In a study from Iran, by using a multiplex PCR, *N. gonorrhoeae* was detected in 31 (46.0%), *C. trachomatis* in 15 (22.4%), and mixed two infections in 7 (10.4%) urine samples from patients with urethritis [16].

In this study the frequency of *T. vaginalis* infection was 32.7% in the studied population. This is higher than our previous report on the general population of the same region [23]. One explanation might be that this study included women with genital complaints. It is believed that in central provinces of Iran, including Qom and the capital Tehran, the prevalence of *T. vaginalis* infection is higher than other regions [29]. Current data shows that *T. vaginalis* infection was detected in significantly higher number of specimens by conventional PCR compared to wet mount examination (96/98 *vs* 34/98). Since usually tiny drops of liquid culture or vaginal discharge are examined under the microscope, the sensitivity of microscopy is low. We found that the sensitivity of multiplex PCR was more than uniplex for *T. vaginalis,* and higher number of specimens tested positive by multiplex PCR compared to conventional PCR (98/98 *vs* 96/98). One explanation might be that the enzyme used in multiplex PCR was more efficient with higher unit activity than enzyme used in conventional PCR. The higher sensitivity of nucleic acid amplification tests over smear or culture for genital infections has been evidenced in several publications [30]. In one investigation, the urine and vaginal specimens of women were rechecked for *T. vaginalis* detection by PCR and results showed that out of 161 negative samples by direct smear and culture, 7 samples (4.3%) turned positive by PCR [31].

For *C. trachomatis* infection, PCR assay is more sensitive than cell culture [32] and is able to detect all the serotypes of *C. trachomatis* [33]. It has been shown that culture and immunoassay have sensitivity of as low as 55% to 85% for *C. trachomatis* [34]. In a previous study we found that antigen based enzyme

immunoassay for *C. trachomatis* has 59.5% sensitivity [21]. In the present study, prevalence rate of *C. trachomatis* was 11.67% by PCR, while ienzume immunoassay demonstrates prevalence rate of 7.14% in women of the same region [21].

In this study, false detection of four non-gonorrhea Neisseria species in multiplex-PCR reduced the specificity. Given the great similarity of nucleic acid sequences of bacteria of the genus Neisseria, it is very difficult to find specific primers for gonorrhea and many tests, even commercialized, are nonspecific. However, low frequency of successful culture gave PCR a priority for detection of gonorrhea [35].

Conclusions

Present multiplex PCR assay showed same specificity and equivalent or higher sensitivity compared to uniplex PCR and other routine assays. Multiple infections are difficult to detect, because a few clinics and laboratories perform diagnoses to *N. gonorrhoeae, C. trachomatis* and *T. vaginalis* and some centers may not look for a second STI after one is detected. The introduction of multiplex PCR offers a sensitive and more rapid method for the detection of multiple pathogens involved in STIs in a single specimen. Additional studies performed with larger numbers of women, symptomatic and asymptomatic men, and other specimens such as urine will be required to determine the true performance of this multiplex PCR for detection of *T. vaginalis, C. trachomatis* and *N. gonorrhoeae* simultaneously.

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Authors' contributions

MNR designed and supervised the study, performed data analysis and wrote the paper. BHR helped in samples collection and volunteers' examination. MNR helped in development of protocols and establishment of the methods. FA and AH performed the laboratory experiments.

References

1. World Health Organization (2013) Sexually transmitted infections (STIs) Available: http://www.who.int/mediacentre/factsheets/fs110/en/. Accessed: 25 July 2014.
2. Hirsl-Hecej V, Pustisek N, Sikanic-Dugic N, Domljan LM, Kani D (2006) Prevalence of chlamydial genital infection and associated risk factors in adolescent females at an urban reproductive health care center in Croatia. Coll Antropol 30 Suppl 2: 131-137.
3. Da Ros CT, Schmitt Cda S (2008) Global epidemiology of sexually transmitted diseases. Asian J Androl 10: 110-114.
4. Rashidi BH, Chamani-Tabriz L, Haghollahi F, Jeddi-Tehrani M, Naghizadeh MM, Shariat M, Akhondi MM, Bagheri R, Asgari S, Wylie K (2013) Effects of *Chlamydia trachomatis* infection on fertility; a case-control study. J Reprod Infertil 14: 67-72.
5. Price MJ, Ades AE, De Angelis D, Welton NJ, Macleod J, Soldan K, Simms I, Turner K, Horner PJ (2013) Risk of pelvic inflammatory disease following *Chlamydia trachomatis* infection: analysis of prospective studies with a multistate model. Am J Epidemiol 178: 484-492.
6. Hay P, Ugwumadu A (2009) Detecting and treating common sexually transmitted diseases. Best Pract Res Clin Obstet Gynaecol 23: 647-660.
7. Dean D, Turingan RS, Thomann HU, Zolotova A, Rothschild J, Joseph SJ, Read TD, Tan E, Selden RF (2012) A multiplexed microfluidic PCR assay for sensitive and specific point-of-care detection of *Chlamydia trachomatis*. PLoS One 7: e51685.
8. Tang J, Zhou L, Liu X, Zhang C, Zhao Y, Wang Y (2011) Novel multiplex real-time PCR system using the SNP technology for the simultaneous diagnosis of *Chlamydia trachomatis*, *Ureaplasma parvum* and *Ureaplasma urealyticum* and genetic typing of serovars of *C. trachomatis* and *U. parvum* in NGU. Mol Cell Probes 25: 55-59.
9. O'Neil D, Doseeva V, Rothmann T, Wolff J, Nazarenko I (2011) Evaluation of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* detection in urine, endocervical, and vaginal specimens by a multiplexed isothermal thermophilic helicase-dependent amplification (tHDA) assay. J Clin Microbiol 49: 4121-4125.
10. McKechnie ML, Hillman R, Couldwell D, Kong F, Freedman E, Wang H, Gilbert GL (2009) Simultaneous identification of 14 genital microorganisms in urine by use of a multiplex PCR-based reverse line blot assay. J Clin Microbiol 47: 1871-1877.
11. Whiley DM, Sloots TP (2005) Comparison of three in-house multiplex PCR assays for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* using real-time and conventional detection methodologies. Pathology 37: 364-370.
12. Patel AL, Sachdev D, Nagpal P, Chaudhry U, Sonkar SC, Mendiratta SL, Saluja D (2010) Prevalence of Chlamydia infection among women visiting a gynaecology outpatient department: evaluation of an in-house PCR assay for detection of *Chlamydia trachomatis*. Ann Clin Microbiol Antimicrob 9: 24.
13. Shipitsyna E, Zolotoverkhaya E, Agne-Stadling I, Krysanova A, Savicheva A, Sokolovsky E, Domeika M, Unemo M (2009) First evaluation of six nucleic acid amplification tests widely used in the diagnosis of *Chlamydia trachomatis* in Russia. J Eur Acad Dermatol Venereol 23: 268-276.
14. Olshen E, Shrier LA (2005) Diagnostic tests for chlamydial and gonorrheal infections. Semin Pediatr Infect Dis 16: 192-198.
15. Ginocchio CC, Chapin K, Smith JS, Aslanzadeh J, Snook J, Hill CS, Gaydos CA (2012) Prevalence of *Trichomonas vaginalis* and coinfection with *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in the United States as determined by the Aptima *Trichomonas vaginalis* nucleic acid amplification assay. J Clin Microbiol 50: 2601-2608.
16. Fathollah zadeh B, Mirsalehian A, Arshadi H, Pourakbari B, Kazemi B (2004) Diagnosis of *Chlamydia trachomatis* and *Neisseria gonhorea* in genitourinary specimens of patients with

urethritis by using PCR and multiplex-PCR. Tehran Univ Med J 62: 449-456.
17. Hopkins MJ, Ashton LJ, Alloba F, Alawattegama A, Hart IJ (2010) Validation of a laboratory-developed real-time PCR protocol for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in urine. Sex Transm Infect 86: 207-211.
18. Lee SR, Chung JM, Kim YG (2007) Rapid one step detection of pathogenic bacteria in urine with sexually transmitted disease (STD) and prostatitis patient by multiplex PCR assay (mPCR). J Microbiol 45: 453-459.
19. Abou Tayoun AN, Burchard PR, Caliendo AM, Scherer A, Tsongalis GJ (2015) A multiplex PCR assay for the simultaneous detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*. Exp Mol Pathol 98: 214-218.
20. Nateghi Rostami M, Khamesipour A, Eskandari SE, Miramin Mohammadi A, Sarraf Nejad A, Keshavarz H (2008) Flow cytometric analysis of *Leishmania* reactive CD4+/CD8+ lymphocyte proliferation in cutaneous leishmaniasis. Iran J Parasitol 3: 9-18.
21. Nateghi Rostami M, Hossein Rashidi B, Aghsaghloo F, Nazari R (2016) Comparison of clinical performance of antigen based-enzyme immunoassay (EIA) and major outer membrane protein (MOMP)-PCR for detection of genital *Chlamydia trachomatis* infection. Int J Reprod Biomed 14: 411-20.
22. Van Dyck E, Ieven M, Pattyn S, Van Damme L, Laga M (2001) Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* by enzyme immunoassay, culture, and three nucleic acid amplification tests. J Clin Microbiol 39: 1751-1756.
23. Nateghi Rostami M, Hossein Rashidi B, Habibi A, Nazari R, Dolati M (2017) Genital infections and reproductive complications associated with *Trichomonas vaginalis, Neisseria gonorrhoeae, and Streptococcus agalactiae* in women of Qom, central Iran. Int J Reprod Biomed 15: 357-366.
24. Mahony JB, Luinstra KE, Tyndall M, Sellors JW, Krepel J, Chernesky M (1995) Multiplex PCR for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in genitourinary specimens. J Clin Microbiol 33: 3049-3053.
25. Fernandez G, Martro E, Gonzalez V, Saludes V, Bascunana E, Marco C, Rivaya B, López E, Coll P, Matas L, Ausina V (2016) Usefulness of a novel multiplex real-time PCR assay for the diagnosis of sexually-transmitted infections. Enferm Infecc Microbiol Clin 34: 471-476.
26. Kriesel JD, Bhatia AS, Barrus C, Vaughn M, Gardner J, Crisp RJ (2016) Multiplex PCR testing for nine different sexually transmitted diseases. Int J STD AIDS 27: 1275-1282.
27. Mahony JB, Jang D, Chong S, Luinstra K, Sellors J, Tyndall M, Chernesky M (1997) Detection of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, *Ureaplasma urealyticum*, and *Mycoplasma genitalium* in first-void urine specimens by multiplex polymerase chain reaction. Mol Diagn 2: 161-168.
28. Hayakawa T, Mitsuya H, Kojima M, Hayase Y (2002) The clinical evaluation of 414 cases of male urethritis. Nihon Hinyokika Gakkai Zasshi 93: 450-456. [Article in Japanese]
29. Hezarjaribi HZ, Fakhar M, Shokri A, Teshnizi SH, Sadough A, Taghavi M (2015) *Trichomonas vaginalis* infection among Iranian general population of women: a systematic review and meta-analysis. Parasitol Res 114: 1291-1300.
30. Matini M, Rezaie S, Mohebali M, Maghsood AH, Rabiee S, Fallah M, Rezaeian M (2014) Genetic identification of *Trichomonas vaginalis* by using the actin gene and molecular based methods. Iran J Parasitol 9: 329-335.
31. Valadkhani Z, Kazemi F, Assmar M, Amirkhani A, Esfandeari B, Lotfi M, Ghobadirad S, Hassan N, Aghighi Z (2010) Molecular diagnosis of trichomoniasis in negative samples examined by direct smear and culture. Iran J Parasitol 5(4): 31-36.
32. George JA, Panchatcharam TS, Paramasivam R, Balasubramanian S, Chakrapani V, Murugan G (2003) Evaluation of diagnostic efficacy of PCR methods for *Chlamydia trachomatis* infection in genital and urine specimens of symptomatic men and women in India. Jpn J Infect Dis 56: 88-92.
33. Bosnjak Z, Dzijan S, Pavlinic D, Peric M, Ruzman N, Krizan IR, Lauc G, Antolović-Požgain A, Burazin J, Vuković D (2012) Distribution of *Chlamydia trachomatis* serotypes in clinical urogenital samples from north-eastern Croatia. Curr Microbiol 64: 552-560.
34. Puolakkainen M, Hiltunen-Back E, Reunala T, Suhonen S, Lahteenmaki P, Lehtinen M, Paavonen J (1998) Comparison of performances of two commercially available tests, a PCR assay and a ligase chain reaction test, in detection of urogenital *Chlamydia trachomatis* infection. J Clin Microbiol 36(6): 1489-1493.
35. Jahan F, Shamsuzzaman SM, Akter S (2014) Diagnosis of common bacterial causes of urethritis in men by Gram stain, culture and multiplex PCR. Malays J Pathol 36: 175-180.

Corresponding author
Mahmoud Nateghi Rostami, PhD
Department of Microbiology and Immunology, Faculty of Medicine, Qom University of Medical Sciences, Qom, Iran
P.O. Box 14515-6383 Tehran, Iran
Phone: +98-2188970657
Fax: +98-2188963804
Email: Rostami52@yahoo.com

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